CHEMICAL MEDIATION IN CRUSTACEANS. III. ACETYLCHOLINE AND AUTOTOMY IN PETROLISTHES ARMATUS (GIBBES)

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Acetylcholine has been shown to be present in nervous tissues of crustaceans in considerable amounts (Welsh, 1938; Smith, 1939) and to have an excitatory action on the decapod heart (Welsh, 1939 *a* and *b*), but, thus far, its action elsewhere in crustaceans has not been demonstrated (see Katz, 1936, regarding acetylcholine and crustacean skeletal muscle).

Autotomy, or the casting of legs by crustaceans, is a well-known phenomenon resulting from a unisegmental, reflex stimulation of the autotomizer muscle of a leg (Wood and Wood, 1932). Hence central transmission at synapses, peripheral transmission at myoneural junctions, and conduction along fibers of the reflex pathway must occur in this process. If acetylcholine is normally involved in the transmission of nerve impulses in the autotomy reflex, it should be possible to obtain evidence by appropriate injections of acetylcholine and of drugs which are known to affect its rate of destruction and its action. That stimulating and inhibiting substances may play a part in autotomy in *Porcellana* and *Uca* was suggested by Hoadley (1934, 1937). Abramowitz and Abramowitz (1938), while testing a series of drugs for their effects on the chromatophores of *Uca,* observed that the injection of acetylcholine caused autotomy.

The present paper records observations made on autotomy in an anomuran of Bermuda. The experimental work was done at the Bermuda Biological Station and was aided by grants from the Milton and Porter Funds of Harvard University.

Materials and Methods

The animal used was *Petrolisthes armatus* (Gibbes), of the family Porcellanidae. The members of this family are said to autotomize with the greatest ease and after the least stimulus of any of the decapod crustaceans (Wood and Wood, 1932). *P. armatus* occurs in considerable abundance under stones between tide levels in Bermuda. They were captured and handled with care in order to prevent autotomy, and experiments were performed, whenever possible, on animals which had been in the laboratory less than twenty-four hours.

Since *Petrolisthes* may be caused to autotomize a leg by grasping it gently with forceps, this was the method of stimulation employed. Individuals were placed in small containers filled with sea water. Each leg was seized, held for approximately one second, and, if not dropped, was then released. *Petrolisthes* has three pairs of legs which are developed for walking, and a pair of chelae, so a series of eight stimuli could be given the animal without repeated stimulation of those appendages which failed to autotomize on the first trial. Legs were grasped in random order and the order was varied from animal to animal.

After obtaining results on one hundred normal animals to serve as controls, fifty animals were injected at the base of one of the last walking legs with 0.05 cc. of a perfusion fluid prepared according to Pantin (1934). As the average body volume of the animals used was only 0.3 cc., this amount of injected fluid caused a considerable dilution of the blood, but this dilution and the act of injection had no evident effect on autotomy. The drugs employed were always given in the volume of fluid used in the injected controls. These drugs were acetylcholine chloride (Hoffman-LaRoche), eserine (physostigmine) sulphate (Merck), atropine sulphate (Merck), and adrenalin chloride (Parke-Davis). Fresh solutions of these substances were prepared daily and the necessary dilutions were made just before using. This is especially important in the case of acetylcholine which is rapidly hydrolyzed in an alkaline solution.

Results

Control Experiments.—The results obtained on uninjected control animals are given in Fig. 1. The first leg [1] stimulated by grasping was dropped by 54 of the 100 animals, or it may be said that there was 54 per cent autotomy. The second, third and fourth legs stimulated showed increasing tendency to autotomize up to 85 per cent. Then an abrupt drop in the number of autotomies appeared until the eighth, or last leg, autotomized in only 21 per cent of the trials. This increasing tendency to autotomize up to a certain point, followed by a decreasing tendency, is not due to individual differences in the autotomizing mechanisms of different legs, for with such differences alone a varying, random order of stimulation would give a straight line parallel to the abscissa. It

[1] No distinction will be made between walking legs and chelae and both types of appendages will be spoken of as legs.

can be explained, however, by assuming that each stimulus to a leg, whether the leg is dropped or not, affects the tendency of the other legs to autotomize. Up to the fourth stimulus the effect is excitatory and after the fourth stimulus it is inhibitory.

Fifty *Petrolisthes* injected with 0.05 cc. of "crab Ringer" and stimulated ten to fifteen minutes after injection autotomized in a manner similar to the uninjected controls (Fig. 1).

The line which is drawn through the data shown in Fig. 1 may be called the normal curve of autotomy for *Petrolisthes,* when stimulation

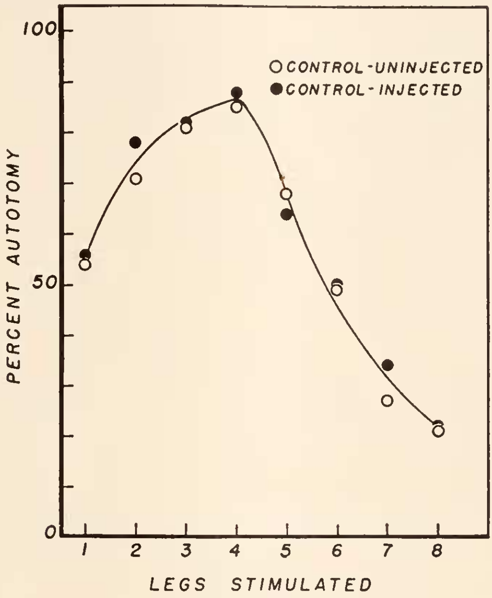

FIG. 1. Percentage of legs dropped by 100 normal animals when each leg was stimulated once, and the order of stimulation was varied from one animal to the next, is shown by the open circles. Results obtained on 50 animals injected with 0.05 cc. of "crab Ringer" are shown by the closed circles. The line drawn through the data has been called the "normal curve" of autotomy.

is as described. An obvious change in the "normal curve" produced by the injection of some substance would give an indication of the effect of that substance on the process of autotomy.

Acetylcholine.—The injection of various amounts of acetylcholine in preliminary tests indicated that 25 gamma of acetylcholine [2] was intermediate between a lethal dose and one whose effects had largely disappeared between the time of injection and the time of stimulation. In

[2] The amount of substance injected will be expressed as the weight of the salt even though the salt is not indicated. The volume of fluid was always 0.05 cc.

most cases larger amounts caused a number of legs to be dropped during the injection process, or shortly after the injection was made. The legs dropped were almost always on the side on which the injections were made.[3] In 20 per cent of the animals the injection of 25 gamma of acetylcholine caused one to three legs to be dropped without further stimulation.

Fifty animals, each of which was injected with 25 gamma of acetylcholine and its legs stimulated approximately five minutes after injection, yielded results which are shown in Fig. 2. The first leg to be grasped

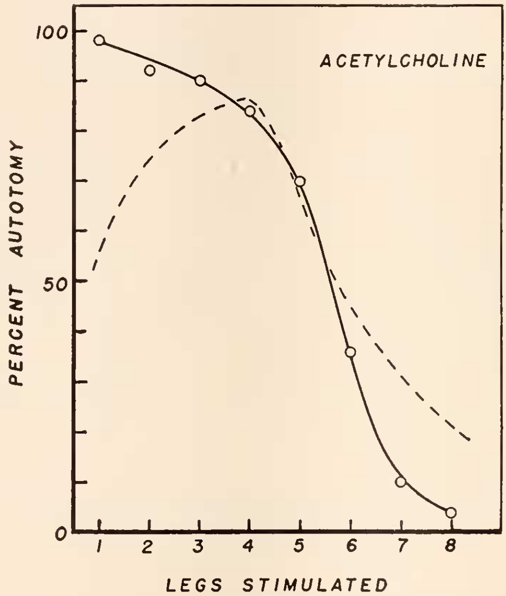

Fig. 2. Percentage autotomy in 50 animals each of which received 25 gamma of acetylcholine chloride five minutes before stimulation of the legs. The dashed line in this and the following figures is the "normal curve" of autotomy.

was dropped in 98 per cent of all the animals, but immediately an "inhibitory" phase appeared and the entire curve resembles the inhibitory part of the "normal curve." It is apparent that the injection of acetylcholine markedly affects the autotomizing process and, at this concentration, eliminates the period of increasing tendency to autotomize.

Eserine.—*Petrolisthes* was found to be remarkably sensitive to eserine. The injection of 0.5 gamma or more caused, after a delay of one

[3] The injection of 100 gamma of acetylcholine into each of six *Pachygrapsus transversus* (Gibbes), a rock crab, caused the autotomy of three to five legs, on the side of the injection, during the injection process. In two cases all of the legs on the opposite side were autotomized within a few seconds after injection.

to two minutes, extreme activity followed by tetanic convulsions and death. The injection of 0.25 gamma caused greatly increased general activity and uncoördinated movements followed by sluggishness, failure to autotomize, and the death of some animals. A dose of 0.1 gamma caused increased activity for a few minutes after injection, but following this the reactions of the majority of the animals appeared normal. This amount was administered to fifty animals in groups of ten, and ten to fifteen minutes later their legs were stimulated as were those of the controls. The results may be seen in Fig. 3. The eserine curve is quite

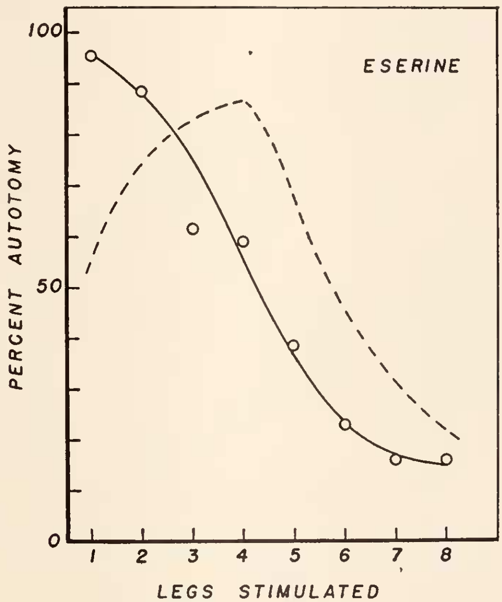

FIG. 3. Percentage autotomy in 50 animals injected with 0.1 gamma of eserine sulphate and their legs stimulated ten to fifteen minutes after injection.

similar to the one obtained for acetylcholine, except that it is slightly lower and shifted somewhat to the left due to a lower percentage of autotomies in all except the last two legs to be stimulated. The important points, however, are the absence of a period of increasing tendency to autotomize, and the large number of autotomies (95 per cent) of first legs to be stimulated.

Atropine.—Relatively large doses of atropine had to be injected before definite effects were obtained. Amounts above 100 gamma caused marked sluggishness and prevented all autotomies during a period of fifteen minutes after injection. A dose of 100 gamma resulted in decreased general activity for a period of five minutes after the injection but ten minutes later the animals appeared normal, hence this was selected as the dose to be employed. Eighty animals were injected in lots of ten, and ten minutes later their legs were stimulated. Fifteen animals failed to drop any legs and in the calculations of percentage of autotomies these were omitted. The remaining sixty-five animals showed a marked reduction in the total number of legs dropped, as may be seen in Fig. 4. Only 46 per cent dropped a leg at the first stimulus and this was followed by a decline in the number of autotomies with

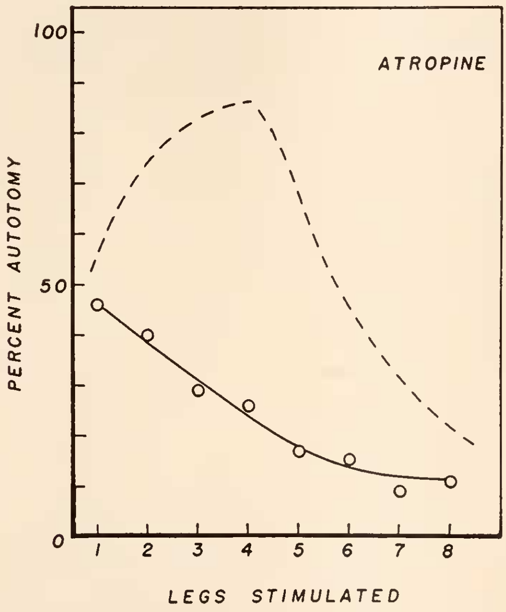

FIG. 4. Percentage autotomy in 65 animals injected with 100 gamma of atropine sulphate and their legs stimulated ten minutes after injection.

subsequent stimuli. Thus atropine inhibits autotomy more or less completely, depending on the amount injected.

Adrenalin.—Adrenalin (adrenine) is known to have an excitatory action on the decapod heart (Bain, 1929; Welsh, 1939*a* and *b*), therefore it seemed desirable to test its effects on autotomy in *Petrolisthes.* Adrenalin chloride was injected in amounts varying from 1 gamma to 50 gamma per animal. A dose of 50 gamma produced marked inactivity, uncoördinated movements and occasionally death. A dose of 1 gamma had no noticeable effects, either on general behavior, or on autotomy. Fifty animals injected with 2.5 gamma of adrenaline and stimulated ten to fifteen minutes later gave results which are shown in Fig. 5. The shape of the curve is not very different from the " normal curve," but its displacement indicates that adrenalin, in this concentration, has a slight inhibitory effect over the entire series of legs stimulated.

Autotomy in Males and Females.—The following observations have only an indirect bearing on the general question of chemical mediation and autotomy, but seem worthy of mention. Evidence presented by Hoadley (1934; 1937) indicated that in *Uca* and *Porcellana,* females without eggs and males tended to autotomize more readily than did females with eggs. Our data on control animals, including one group of one hundred animals which have not been mentioned previously, were examined to see if *Petrolisthes* showed similar differences. The great majority of the females were carrying eggs at the time our observations were made; hence the results for the small group not carrying eggs are of little significance. Table I includes the data from 249 animals. It is apparent from these data that the tendency to autotomize in *Petrolisthes* is not modified by the carrying of eggs. However, this does not preclude the possibility of there being such a modification in other groups of crustaceans.

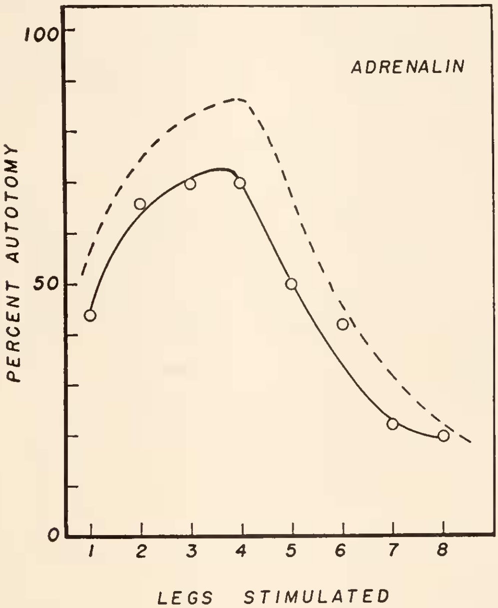

FIG. 5. Percentage autotomy in 50 animals injected with 2.5 gamma of adrenalin chloride and their legs stimulated ten to fifteen minutes after injection.

Discussion

It has been shown (1) that stimulation of the legs of *Petrolisthes armatus* produces changes in the animal which result in an increasing percentage of autotomies for the first four legs followed by a progressive decrease in the number of autotomies for the remaining legs; (2) that the injection of acetylcholine facilitates or causes autotomy; (3) that eserine, which prevents the enzymic hydrolysis of acetylcholine, produces certain effects similar to those produced by injected acetylcholine; (4) that atropine, which prevents the muscarin-like action of acetylcholine, causes a partial or complete reduction in the number of autotomies. This evidence indicates that acetylcholine normally acts in the autotomy reflex of *Petrolisthes.* Interesting questions are: (1) where and in what way does this action occur, and (2) why does the stimulation and autotomy of a leg affect the tendency of remaining legs to be dropped? These and other questions cannot be definitely answered with the evidence at hand but certain suggestions, to be tested by further experiments, may be offered.

TABLE I

	Males		Females with eggs		Females without eggs	
	No. of animals	Average no. legs dropped per animal	No. of animals	Average no. legs dropped per animal	No. of animals	Average no. legs dropped per animal
Regular order of stimulation	52	4.50	45	4.38	3	5.0
Random order of stimulation	49	4.44	48	4.95	3	5.0
Injected controls	23	4.74	22	5.13	4	5.0

Although the ventral ganglia of *Petrolisthes* are fused into a single mass, as in the Brachyura, there is an anatomical arrangement according to a segmental pattern. Stimulation of a leg by pinching excites sensory fibers to the ventral ganglion supplying that segment. Internuncial neurones carry the impulses to the motor fibers which innervate the autotomizer muscle of that leg. Since it is extremely rare for any leg to be dropped other than the one being stimulated, it is apparent that any impulses going out from a ganglion to other ganglia must ordinarily be below threshold for the autotomizer muscles of other legs. However, sub-threshold impulses might produce facilitation, as has been shown by

Pantin (1934; 1936*a* and *b*) for leg muscles of *Carcinus,* and the facilitating process might be the release of acetylcholine at motor nerve endings. There is good evidence that acetylcholine is present in crustacean blood in considerable amounts (Smith, 1939). Its accumulation, as a result of successive stimuli, might account for the regular increase in the number of autotomies of the first four legs stimulated in the control animals. Why, with subsequent stimuli, there should then be a regular decrease in the number of autotomies of the remaining legs is not readily explainable. In accounting for certain phenomena associated with Wedensky inhibition and fatigue in vertebrate muscle, Rosenblueth and Morison (1937) have postulated that high concentrations of acetylcholine are paralytic. Possibly after the fourth leg of *Petrolisthes* is stimulated the acetylcholine concentration in the blood reaches a paralytic range and inhibition then sets in. However, if this were the case, on the basis of the control data one would expect that 85 per cent autotomies would be the maximum obtainable before the paralytic threshold of acetylcholine were reached; instead, injections of acetylcholine and eserine may result in as high as 98 per cent autotomies.

Rosenblueth and Morison (1937) have suggested that in high frequency stimulation of vertebrate nerve the quantal yield of acetylcholine, per impulse, is greatly reduced as stimulation is continued. A similar situation may occur in *Petrolisthes* as successive volleys of impulses traverse the reflex pathways. Taking into consideration the rate of production and the rate of destruction of the acetylcholine it follows that its concentration in the general circulation would rise to a maximum followed by a sharp decline as stimulation is continued. If, then, the degree of facilitation of autotomy is directly dependent on the concentration of acetylcholine in the region of the autotomizer muscles, one would expect precisely the type of curves obtained from the control animals and from those injected with acetylcholine. Additional data must be obtained, however, before this explanation is entirely acceptable.

What appears to be a clearly demonstrable rôle of acetylcholine in autotomy in crustaceans is of interest in itself but of greater interest is the evidence that acetylcholine may, after all, be the transmitter or mediator substance between motor nerves and skeletal muscle of crustaceans.

Summary

1. A single stimulus to each of the walking legs and chelae of the anomuran, *Petrolisthes armatus,* was found to cause, in most animals, the autotomy of four or more of these eight appendages. The appendages least often dropped were the first, sixth, seventh, and eighth of a series, when the order of stimulation was a random one. A regular

increasing tendency to autotomize in the first four legs was followed by a decreasing tendency in the remainder.

2. Injection of acetylcholine in relatively high concentrations caused the autotomy of one to several legs without further stimulation. A concentration was found which only occasionally caused autotomy when injected, but which facilitated the autotomy of the first three legs to be stimulated and eliminated, thereby, the period of increasing tendency to autotomize.

3. The injection of eserine caused a marked increase in general activity and a relatively small amount (0.1 gamma per animal) facilitated the autotomy of the first and second legs. The injection of eserine never caused legs to be dropped unless they were stimulated, otherwise its effects on autotomy were very similar to those produced by acetylcholine.

4. Atropine caused a general lowering of excitability and in relatively high concentrations completely prevented autotomy.

5. The injection of adrenalin was followed by a reduction in the percentage of autotomies of each of the eight legs but the phases of increasing and decreasing " excitability " remained unchanged.

6. No differences, correlated with sex or the bearing of eggs, were found in the average number of autotomies per individual.

7. The evidence obtained has been interpreted as indicating that acetylcholine normally plays a rôle in the autotomy reflex of *Petrolisthes* probably by acting as a mediator of impulses between the motor nerve and autotomizer muscle of a leg.

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